EVALUATION OF THE ANTIFUNGAL ACTIVITY BY PLANT EXTRACTS AGAINST *Colletotrichum gloeosporioides* PENZ

Avaliação da atividade anti-fúngica de extratos vegetais contra *Colletotrichum gloeosporioides* Penz

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ABSTRACT

Aiming to develop more efficient and environmental friendly methods than those available to control *Colletotrichum gloeosporioides* Penz, which causes blister spot in coffee trees, a search for plants able to produce substances active against such pathogen was carried out. Thus, extracts of 48 plant species, collected at Alto Rio Grande region, in Minas Gerais, were prepared and submitted to *in vitro* assays with that fungus. The best results were obtained with the extracts prepared from *Digitalis lanata* Ehrh, *Origanum manjorona* L., *Plantago lanceolata* Hook. and *Stevia rebaudiana* (Bertoni) Bertoni, which inhibited *C. gloeosporioides* spores germination. After dilution of some active extracts with aqueous 1 % Tween 80 solution in a 1:2 or 1:3 ratio (extract:aqueous solution), their antifungal activity vanished. Some of the active extracts were also submitted to freeze drying and none of them presented any alteration in their antifungal activity. Concluding, several plants presented potential to be used in the search for new bioactive substances to control *C. gloeosporioides*, especially *O. manjorona* L., which inhibited 96 % of the fungus spores germination.

Index terms: Disease control, biocide, Coffee's blister spot.

RESUMO

Com vistas a contribuir para o desenvolvimento de métodos mais eficientes e menos agressivos ao ambiente que aqueles disponíveis para o controle de *Colletotrichum gloeosporioides* Penz, causador da mancha manteigosa em cafeeiros, buscou-se identificar espécies vegetais produtoras de substâncias ativas contra o referido patógeno. Para tanto, prepararam-se extratos de 48 espécies vegetais, coletadas na região do Alto Rio Grande, em Minas Gerais, para serem submetidos a testes *in vitro* com o mencionado fungo. Constatou-se que os melhores resultados foram obtidos com os extratos oriundos de *Digitalis lanata* Ehrh, *Origanum manjorona* L., *Plantago lanceolata* Hook. e *Stevia rebaudiana* (Bertoni) Bertoni que inibiram a germinação dos esporos de *C. gloeosporioides.* Alguns dos extratos ativos também foram submetidos a diluições em solução aquosa de Tween 80 a 1%, o que permitiu observar que, na proporção de 1:2 ou 1:3 (extrato: solução aquosa), os extratos perdiam a atividade antifúngica. Verificou-se também que alguns dos extratos ativos não alteraram suas atividades quando submetidos ao processo de liofilização. Desse modo, identificaram-se várias plantas promissoras na pesquisa por novas substâncias bioativas para o controle de *C. gloeosporioides*, com destaque para *O. manjorona* L., que inibiu 96% da germinação dos esporos do referido fungo.

Termos para indexação: Controle de doenças, biocida, mancha manteigosa do cafeeiro.

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INTRODUCTION

Coffee is one of the most important export crops for Brazil, which is the leading producer and exporter of this commodity. With an area of 2.2 million ha devoted to coffee plants, such country produced 28.82 million sacks of coffee during the year of 2004 and exported the equivalent to US$ 1.51 billions in coffee products at the same year (BRASIL, 2005).

Among the phytopathological problems that occur during the *Coffea arabica* L. cultivation are those caused by fungi like *Colletotrichum* spp., whose presence in several coffee producing Brazilian regions has been constantly reported (CARVALHO, 2004). The symptoms of the fungal parasitism vary in accordance with the species, resulting in different names for the corresponding diseases. One of the most important is the blister spot in coffee plants, which is caused by the

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fungus *Colletotrichum gloeosporioides* Penz (MIRANDA, 2003).

Traditionally, plant fungal diseases are controlled by synthetic fungicides, which increase agricultural costs and contaminate the environment with very toxic substances (CARVALHO, 2004). A possible alternative to solve such problem is the use of plants able to produce antifungal substances (MIRANDA, 2003). Among the several examples available in the literature are *Zanthoxylum americanum* Mill. and *Piper regnellii* var. pallescens (C. DC.) Yunck, whose antifungal activities are well known (BAFI-YEBOA et al., 2005; PESSINI et al., 2005).

Thus, this work aimed to contribute for the developement of new methods to control fungi in coffee plants by the identification of plant extracts active against *C. gloeosporioides*.

MATERIAL AND METHODS

Fresh leaves (3.0 g) of several plants (Table 1) collected in the Alto Rio Grande Region, State of Minas Gerais, Brazil, were cut in small pieces and soaked in methanol during 48 h. The resulting mixtures were filtered and the insoluble parts were soaked in more methanol during the same period of time. The new mixtures were filtered and the liquids obtained during both filtrations were combined and concentrated to dryness in a rotary evaporator. Immediately before carrying out the tests, the dry residues were dissolved in an aqueous 1 % (g/mL) Tween 80 solution (30 mL), resulting in the extracts to be studied.

C. gloeosporioides, isolated by Miranda (2003) and kept on deposit at Departamento de Fitopatologia - Universidade Federal de Lavras, was transferred to Petri dishes containing the culture medium MEA (20 g Agar, 20 g malt extract and 1 L distilled water), which was previously autoclaved at 120 °C. After seven days at 22 °C, under 12 h photoperiods, sterilized distilled water was poured into the dishes and, with a sterilized small paintbrush, the spores were removed. They were counted in the resulting suspension by the use of a Neubauer chamber and water was added to reach 4.0×10^4 spores/mL.

As described by Carvalho (2004), the spore suspension (40 µL) was poured into each well of three well glass slides, which were kept inside Petri dishes. The plant extracts dissolved in aqueous Tween 80 (40 µL) were also poured into the wells and, after seven days at 22°C, which was the best condition for maximum spore germination, 20 mL of a lactoglycerol solution (20 g lactic acid, 40 g glycerol, 20 mL distilled water and 0.05 g trypan blue) was poured into all wells to stop the germination. The wells were divided into five identical parts, in which 20 spores were counted. The percentage of germinated spores in each well was obtained after summing the values of germinated spores in each part.

All tests were carried out with three repetitions, arranged in a randomized design, employing aqueous 1 % Tween 80 solution as control. Statistical calculations were done using SISVAR software (FERREIRA, 2000) and values of germinated spores (%) underwent variance analysis using ANOVA; means were analyzed according to the Scott and Knott test ($P \leq 0.05$). According to Banzatto & Kronka (1989), the observed heterogeneity of variances (9:1) was above the recommended (7:1) for a joint statistical analysis. As a consequence, the experiments underwent statistical calculation separated from each other.

Some of the extracts affording low values of germinated spores were tested again at different concentrations: 1:0, 1:1 and 1:3 (plant extract dissolved in 1% Tween 80: 1% Tween 80). Statistical analysis were done as described above. However, as the homogeneity of variances was observed, a joint statistical calculation was performed.

The most promising extracts were once more prepared from 1.0 g of fresh plant material. Nevertheless, after the solvent removal, half of each residue were freeze-dried before dissolution in aqueous Tween 80 solution. The resulting extracts were submitted to the test with *C. gloeosporioides* as described above and values of germinated spores (%) underwent a non-parametric statistical analysis, employing the Wilcoxon test for matched pairs (SIEGEL, 1977). A *P*-value <0.05 was accepted as statistically significant.

RESULTS AND DISCUSSION

No influence on *C. gloeosporioides* was observed when the corresponding spores were treated with the extracts of *C. jobi*, *C. officinalis*, *D. pectinata*, *H. rosa*, *M. pulegium*, *N. catarica*, *N. tabacum*, *Origanum* sp., *P. sonchifolia* and *T. officinale* (Table 1). This seemed a reasonable result, since no report in the literature was found about the antifungal activity of such plants.

Although the activity against fungi has already been described for the extracts of *A. arborescens*, *H. perforatum*, *J. curcas*, *P. granatum*, *P. graveolens*, *S. officinale*, *S. officinalis* and *S. nigra* (DAMME et al., 2002; GIAMPERI et al., 2002; HOLETZ et al., 2002; KAWAI et al., 1998; LU et al., 2002; SENER, 1994; SHIN, 2003; THANGAVELU et al., 2004), none of them influenced the germination of *C. gloeosporioides* spores (Table 1).

Table 1 – Percentage of *Colletotrichum gloeosporioides* germinated spores after treatment with plant extracts at a constant concentration.

Family	Scientific name	Vernacular name	Germinated spores (%)
Experiment 1			
Poaceae	*Coix lacryma-jobi* L.	Job's tears	62.6 a
Euphorbiaceae	*Jatropha curcas* L.	Barbados nut	69.3 a
Boraginaceae	*Symphytum officinale* L.	Comfrey	67.0 a
	1% Tween 80		68.0 a
Experiment 2			
Asteraceae	*Achillea millefolium* L.	Common yarrow	58.3 b
Asphodelaceae	*Aloe saponaria* (Aiton) Haw	African aloe	50.3 a
Gleicheniaceae	*Dicranopteris pectinata* (Willd.) Und.	Fern	76.0 c
	1% Tween 80		73.6 c
Experiment 3			
Asteraceae	*Achillea millefolium* L.*	Common yarrow	58.6 a
Rosaceae	*Agrimonia eupatoria* L.	Common agrimony	52.6 a
Asteraceae	*Calendula officinalis* L.	English marigold	74.0 a
	1% Tween 80		63.6 a
Experiment 4			
Asteraceae	*Arctium lappa* (Hill) Bernh.	Greater burdock	52.6 a
Asphodelaceae	*Aloe arborescens* Mill.	Krantz aloe	75.6 b
	1% Tween 80		74.0 b
Experiment 5			
Apiaceae	*Foeniculum vulgare* Mill.	Sweet fennel	85.0 c
Lamiaceae	*Glechoma hederacea* L.	Ground ivy	81.3 c
	Mentha pulegium L.	Pennyroyal	68.3 b
	Nepeta cataria L.	Catnip	72.0 b
	Ocimum gratissimum L.	African basil	13.0 a
Geraniaceae	*Pelargonium graveolens* L'Hér	Geranium	70.6 b
	1% Tween 80		67.3 b
Experiment 6			
Lamiaceae	*Mentha spicata* L.	Spearmint	96.6 d
Nyctaginaceae	*Mirabilis jalapa* L.	Four o'clock flower	95.3 d
Moringaceae	*Moringa oleifera* Lam.	Moringa	88.3 d
Rutaceae	*Ruta graveolens* L.	Rue	06.6 a
Lamiaceae	*Thymus vulgaris* L.	Thyme	15.0 b
	1% Tween 80		50.0 c

To be continued...

Table 1 – Continued...

Family	Scientific name	Vernacular name	Germinated spores (%)
Experiment 7			
Clusiaceae	*Hypericum perforatum* L.	St. John's wort	87.3 a
Lamiaceae	*Origanum* sp.		76.6 a
	Salvia officinalis L.	Sage	72.0 a
Caprifoliaceae	*Sambucus nigra* L.	Black elderberry	75.0 a
	1% Tween 80		74.3 a
Experiment 8			
Plantaginaceae	*Plantago lanceolata* Hook.	Narrowleaf plantain	25.3 a
Punicaceae	*Punica granatum* L.	Pomegranate	72.0 b
	1% Tween 80		70.3 b
Experiment 9			
Rubiaceae	*Coffea arabica* L.	Coffee	84.0 c
Rutaceae	*Citrus* sp.		05.3 a
Scrophulariaceae	*Digitalis lanata* Ehrh	Woolly foxglove	18.3 b
Malpighiaceae	*Malpighia glabra* L.	Acerola	95.6 d
Phytolacaceae	*Petiveria alliacea* L.	Anamu	08.3 a
Anacardiaceae	*Schinus molle* L.	Brazillian peppertree	20.6 b
Asteraceae	*Stevia rebaudiana* (Bertoni) Bertoni	Sweetleaf	17.0 b
	1% Tween 80		77.6 c
Experiment 10			
Malvaceae	*Hibiscus rosa sinensis* L.	Chinese hibiscus	96.3 d
Lamiaceae	*Melissa officinalis* L.	Lemon balm	66.3 b
	Origanum majorona L.	Oregano	03.3 a
Asteraceae	*Parthenium hysterophorus* L.	Salvinia	79.0 c
Plantaginaceae	*Plantago major* L.	Common plantain	63.6 b
Asteraceae	*Polymnia sonchifolia* Poepp.	Yacon	90.0 d
	1% Tween 80		91.3 d
Experiment 11			
Rutaceae	*Citrus limon* (L.) Burm. f.	Lemon	01.6 a
Onagraceae	*Oenothera biennis* L.	Common evening primrose	48.6 b
	1% Tween 80		78.6 c
Experiment 12			
Rubiaceae	*Coffea arabica* L. cv. Acaiá-Cerrado MG1474)	Coffee	84.3 a
Zingiberaceae	*Curcuma longa* L.	Turmeric	87.3 a
	1% Tween 80		97.3 a

To be continued...

Table 1 – Continued...

Family	Scientific name	Vernacular name	Germinated spores (%)
Experiment 13			
Zingiberaceae	*Curcuma zedoaria* (Christm.) Roscoe	Zedoary	33.3 a
Solanaceae	*Nicotiana tabacum* L.	Tobacco	90.6 c
Phytolacaceae	*Petiveria* sp.		55.3 b
Asteraceae	*Taraxacum officinale* Weber	Common dandelion	90.3 c
	1% Tween 80		88.0 c

Means of six replicates with the same letter do not differ significantly ($P \leq 0.05$). CV values varied between 3.5 and 14.3 %.
*A mixture of leaves and inflorescences was used to prepare the extract.

An unexpected result was obtained for the extracts of *F. vulgare, G. hederaceae, M. glabra, M. spicata* and *M. oleifera*, whose antifungal activity has already been described in the literature (ABOU-JAWDAH et al., 2002; ADAM et al., 1998; CACERES et al., 1993; CORTEZ et al., 1998; LIMA et al., 1992; PATRA et al., 2002). Somehow, they increased the germination of *C. gloeosporioides* spores (Table 1).

The extracts of *A. lappa, A. millefolium, Citrus* sp., *C. limon, C. longa, C. zedoarea, M. officinalis, O. biennis, O. gratissimum, P. alliacea, P. hysterophorus, P. major, R. graveolens* and *T. vulgaris* were all active against *C. gloeosporioides* (Table 1), what is in accordance with the activity previously described against fungi from other genus (BENEVIDES et al., 2001; FICKER et al., 2003; FIORI et al., 2000; GIAMPERI et al., 2002; HOLETZ et al., 2002; KISHORE et al., 1996; MISHRA et al., 2002; NINO et al., 2003; POPOVIC et al., 2002; ROTH et al., 1998; SHRIVASTAVA et al., 1984; SHUKLA et al., 1999; SOUZA et al., 2005; TIWARI et al., 2003). Specifically for *R. graveolens*, the activity of the corresponding extract against *C. gloesporioides* was already known (OLIVA et al., 2003).

The extract of *C. arabica* cv. Acaiá-Cerrado MG1474 was also studied, since this coffee plant was less susceptible to *C. gloeosporioides* (FERREIRA, 2004) than other cultivars. Nevertheless, only a weak inhibition of spore germination was observed (Table 1).

A. eupatoria and *Petiveria* sp. extracts presented moderate antifungal action, while those from *D. lanata, P. lanceolata* and *S. rebaudiana* afforded low values of germinated spores. One of the best results was observed for the extract of *O. manjorona*, which inhibited about 96 % of the spores. These results were a surprise, since no report about the antifungal activity of such plants was found.

In order to assess the effect of extracts concentration on *C. gloeosporioides* spores, six of them were randomly selected among extracts presenting antifungal activity. None of them influenced spore germination in a 1:3 ratio (extract dissolved in 1 % Tween 80: 1% Tween 80)(Table 2).

The amount of germinated spores at 1:0 ratio (extract dissolved in 1 % Tween 80: 1% Tween 80) was approximately twice the value presented in Table 1 for those extracts prepared from de *R. graveolens, S. rebaudiana* and *T. vulgaris*. Regarding *O. manjorona*, the difference between values shown in Table 1 and Table 2 was much smaller, but for *Citrus* sp. the percentage of germinated spores was about four times less in the former Table. Probably, it ocurred due to differences in the plant collecting period, which can influence metabolite production (SAITO et al., 2004).

To simplify the purification process to be employed in the future to isolate the active substances and to preserve them, it is desirable to eliminate humidity. One of the softest processes to do so is freeze-drying (PASTORINI et al., 2002), whose influence on plant extract activity was evaluated in this work. With a probability of 0.109 and a level of significance fixed in 5%, it became clear after the Wilcoxon test that the freeze-drying process did not affect the antifungal activity (Table 3).

Table 2 – Percentage of *Colletotrichum gloeosporioides* germinated spores after treatment with plant extracts at different concentrations.

	Germinated spores (%)		
Plant	100% Extract	50% Extract	25% Extract
Citrus sp.	23.6 c	98.3 c	97.6 a
Ocimum gratissimum	0.6 a	88.0 b	97.0 a
Origanum manjorona	4.3 a	85.3 b	94.0 a
Ruta graveolens	14.3 b	85.3 b	90.0 a
Stevia rebaudiana	42.3 d	98.6 c	98.6 a
Thymus vulgaris	31.3 c	74.0 a	91.6 a
1% Tween 80	92.5 e	92.0 c	92.0 a
CV(%)=5.57			

Means of three replicates with the same letter in a column do not differ significantly ($P \leq 0.05$)

Table 3 – Percentage of *Colletotrichum gloeosporioides* germinated spores after treatment with plant extracts before and after freeze-drying.

Plant	Part	Geminated spores (%)		*d*	Posto de *d*
		Before	After		
Citrus limonia	Leaf	0.0	2.0	-2.00	-9.5
Citrus sp.	Leaf	0.0	0.3	-0.33	-4.0
Digitalis lanata	Flower	0.0	0.6	-0.66	-6.0
Digitalis lanata	Leaf	0.0	1.0	-1.00	-7.0
Ocimum gratissimum	Flower	3.7	0.0	3.67	12.0
Ocimum gratissimum	Leaf	0.0	0.0	0.00	2.0
Origanum manjorona	Leaf	0.0	0.0	0.00	2.0
Petiveria alliacea	Leaf	0.0	2.7	-2.67	-11.0
Ruta graveolens	Leaf	0.0	1.3	-1.33	-8.0
Stevia rebaudiana	Flower	2.0	4.0	-2.00	-9.5
Stevia rebaudiana	Leaf	0.6	1.0	-0.34	-5.0
Thymus vulgaris	Leaf	0.0	0.0	0.00	2.0
T=18					

Statistical analysis was carried out with the Wilcoxon test for matched pairs.

CONCLUSION

Among the several extracts studied, those from *A. eupatoria*, *Petiveria* sp. and mainly from *D. lanata*, *P. lanceolata* and *S. rebaudiana*, afforded very promissing results to be used for the control of *C. gloeosporioides*. The most active extract was that from *O. manjorona*, which inhibited 96 % of *Colletotrichum gloeosporioides* spore germination.

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